Introduction

1. INTRODUCTION

1.1 Diabetes

Diabetes is a chronic disease that has two common forms, diabetes insipidus and diabetes mellitus that arises when the pancreas do not produce enough insulin, or when the body cannot effectively use the insulin it produces. Failure to produce insulin, or insulin to act properly, or both, leads to raised glucose levels in the blood (hyperglycemia). This is associated with long-term damage to the body and failure of various organs and tissues. Diabetes is a polygenic phenomenon which has both genetic and environmental etiological factors. Commonly certain ethnic groups such as Polynesian, American Indians and Hispanics etc show genetic predisposition to diabetes. Environmental predisposing factors include family history, small birth weight, history of glucose intolerance, gestational diabetes and certain syndromes such as polycystic ovarian syndrome.[1,2]

Data from global studies demonstrates that the number of people with diabetes in 2011 has reached a staggering 366 million and it is estimated that by 2030 one adult in ten will have diabetes[3].

1.1.1 Types of diabetes

There are three main type of diabetes[3]:

A. **Type-1 diabetes:** It is also called insulin-dependent, immune-mediated or juvenile-onset diabetes. The disease can affect people of any age, but usually occurs in children or young adults. It is caused by an auto-immune reaction where the body's defense system attacks the β-cells of pancreas. The reason for this autoimmunity is not fully understood. People with this form of diabetes (Type-1) need insulin injection in order to control the levels of glucose in their blood.

B. **Type-2 diabetes:** It is also called non-insulin dependent diabetes or adult-onset diabetes and accounts for at least 90% of all cases of diabetes. The diagnosis of type-2 diabetes usually occurs after the age of 40 but can occur earlier, especially in high diabetes prevalence populations. It is characterized by insulin

resistance and relative insulin deficiency, either of which may lead to non-insulin dependent diabetes mellitus (NIDDM).

C. **Gestational diabetes (GDM)**: It develops in one in 25 pregnancies worldwide and is associated with complications in the period immediately before and after birth. It is a form of diabetes consisting of high blood glucose levels during pregnancy. GDM usually disappears after pregnancy but women with GDM and their offspring are at an increased risk of developing type-2 diabetes later in their life. Approximately half of women with a history of GDM go on to develop type-2 diabetes within five to ten years after delivery.

1.1.2 Complications involved in diabetes

Type-1 and Type-2 diabetes is a life-long condition that requires careful monitoring and control of blood glucose. Without proper management they can lead to very high blood glucose levels which can result in long term damage to various organs and tissues (**Figure-1**). The most affected organs are CVS, nervous system, kidneys and eye[2,3].

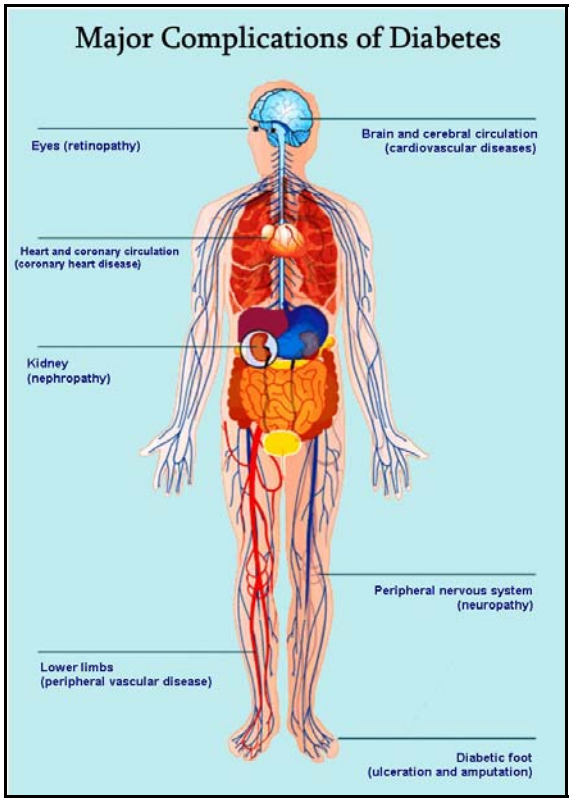

Figure-1: Major complications involved in diabetes[2,3].

A. **Cardiovascular disease:** Cardiovascular disease is the major cause of death in people with diabetes, accounting in most populations for 50% or more of all diabetes fatalities, and much disability. It affects the heart and blood vessels and may cause fatal complications such as coronary heart disease (leading to heart attack) and stroke.

B. **Kidney disease (diabetic nephropathy):** In the USA and other countries diabetes is an increasingly important cause of renal failure and indeed has now become the single most common cause of end stage renal disease, i.e. that which requires either dialysis or kidney transplantation.

C. **Nerve disease (diabetic neuropathy):** Loss of feeling is a particular risk because it can allow foot injuries to escape notice and treatment, leading to major infections and amputation which can ultimately lead to ulceration and amputation of the toes, feet and lower limbs.

D. **Eye disease (diabetic retinopathy):** Characterized by damage to the retina of the eye this can lead to vision loss.

1.1.3 Management of diabetes

Today, there is no cure for diabetes, but effective treatment exists (**Table-1**). Access to appropriate medication, quality of care and good medical advice brings about an active and healthy life reducing the risk of developing complications. This can be achieved by a combination of physical activity, body weight control, healthy eating, avoid of tobacco and effective pharmacotherapy (**Figure-2**)[1,3].

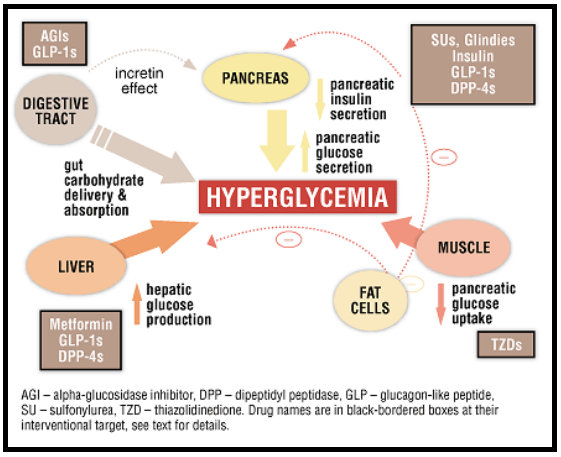

Figure-2: Current pharmacotherapies of diabetes[1,3].

1.1.3.1 Current pharmacotherapy

Typically monotherapy is the first step and to control HbA1c below 7.0%, if it fails combination therapy is implemented. Generally injected insulin is reserved for patients who fail to adequately respond to combination therapy or those with safety concerns such as pregnancy, or severe renal, or liver impairment. Currently there are five mechanistic classes of anti-diabetic agents[1,2] that include:

A. Carbohydrate modulators which delay intestinal absorption of monosaccharides (i.e., α-glucosidase inhibitors),

B. Insulin secretagogues which increase the exocytosis of insulin from β-cells (sulfonylureas and other K_{ATP} channel stimulators such as repaglinide, nateglinide, etc.),

C. Agents that have direct insulin sensitizing effects on peripheral insulin responsive tissues (biguanides such as metformin; peroxisome proliferator-activated receptor gamma (PPARγ) full agonists such as rosiglitazone and pioglitazone),

D. Incretin potentiators (exenatide is an incretin analog and sitagliptin is an inhibitor of incretin degradation), and

E. Amylin analog (pramlintide).

Table-1: Current therapeutic agents for type-2 diabetes

Sr. No.	Drug Class	Molecular target and observed effects	Indication, contraindication, side effects	Available drugs
1	α-glucosidase inhibitors	▪ Inhibit the activity of α-glucosidase ▪ enzymes in the brush border of enterocytes lining the intestinal villiand ▪ Delays the absorption of glucose and alters the release of glucose-dependent intestinal hormones.	Gastrointestinal disturbances	Acarbose Miglitol Voglibose

Sr. No.	Drug Class	Molecular target and observed effects	Indication, contraindication, side effects	Available drugs
2	Insulin secretagogues (Sulfonylureas and other K_{ATP} directed agents)	▪ Bind the sulfonylurea receptor (SUR-1) in β-cells. ▪ SUR-1 binding loses K_{ATP} channels. ▪ Depolarization opens Ca^{++}channels increasing intracellular Ca^{++} levels and facilitates insulin release.	Deterioration of glycemic control over time	Glibenclamide Repaglinide, Nateglinide, etc.
3	Insulin sensitizing effects [biguanides and peroxisome proliferator-activated receptor gamma (PPARγ) agonist]	Insulin sensitizers have direct pleiotropic effects on insulin responsive tissues, generally enhancing insulin sensitivity in these tissues.	Impaired renal function, liver disease, metabolic acidosis, abdominal discomfort and diarrhea	metformin, phenformin, buformin, rosiglitazone and pioglitazone
4	Incretin potentiators GLP-1 Receptor (GLP-1R) Agonist and DPP-IV Inhibitors	Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), stimulate pancreatic β-cells within 10 min of a meal to secrete insulin.		Exenatide and Sitagliptin
5	Amylin analogs	Amylin binds to a G-protein coupled receptor (GPCR) that suppresses glucagon secretion, slows gastric emptying time, and reduces food intake (i.e., satiating effect)	hypoglycemia, nausea and vomiting	Pramlintide
6	Insulin analogs	Suppresses glucose production and augments glucose utilization	hypoglycemia, weight gain	Insulin Zinc suspension, Protamine Zinc suspension

1.1.4 Emerging anti-diabetes targets

Base on the primary type of beneficial effects these targets are roughly divided into four general areas (**Table-2**)[1]

Table-2: Emerging Anti-diabetes Targets

Sr. No.	Type	Class		Site of action
A	Modulators of carbohydrate metabolism/disposition	1	Glucokinase (GK) activators	Convert glucose to glucose-6-phosphate in glycolytic pathway. GK is expressed in neuronal/neuroendocrine cells, hepatocytes and pancreatic β-cells.
		2	Fructose-1,6-bisphosphatase (FBP) inhibitors	Catalyze reversed conversion of Fructose-1,6-Bisphosphatase to Fructose-6-phosphatase in gluconeogenesis in liver.
		3	Glycogen Phosphorylase (GP) inhibitors	Convert glycogen to glucose and produced glucose-1-phosphate by glycogenolysis.
		4	Sodium/Glucose Co-Transporter (SGLT) inhibitors	Allow excretion of glucose in urine and expressed specifically in the intestine and kidneys.
		5	AMP-activated Protein Kinase activators	Act on multiple substrate by deactivating PEPCK and glucose-6-phosphatase and increased glucose uptake by inducing GLUT4 and GLUT1 in muscles.

Sr. No.	Type	Class		Site of action
B	Fat cell and lipid level modulators	1	β_3-Adrenergic Receptor (β_3-AR) agonists	Activation of β_3-AR activates uncoupling protein (UCP) in adipose tissue, which causes the expenditure of metabolic calories as heat.
		2	Hormone Sensitivity Lipase (HSL) inhibitors	HSL allows mobilization of FFA from adipose tissue which allows lipolysis.
		3	Adipocyte Fatty Acid Binding Protein (aFABP) inhibitors	aFABP forms complex with HSL and allows release of FFA's in adipocytes.
		4	GPR40 (Free Fatty Acid Receptor 1 (FFAR1)) ligands	GPR40 preferentially expressed in pancreatic β-cells and islets and mediates the majority of effects of FFA's on insulin secreation.
C	Modulators with pleiotropic effects on carbohydrate,lipid or protein metabolism	1	Retinoic Acid X Receptor (RXR) modulators	Modulators of RXR shown to have glucose-lowering, insulin sensitizing and antiobesity effect
		2	Liver X Receptor (LXR) modulators	LXR is sensor receptor which induces a programme of gene transcription that increases the removal of cholesterol and metabolites.
		3	Farnesoid X Receptor (FXR) modulators	FXR regulates metabolic effects by repression of PEPCK, PPARα, PDK4

Sr. No.	Type	Class		Site of action
		4	Pan agonists, -δ directed agonists and selective peroxisome Proliferator-Activated Receptor (PPAR) modulators (SPPARM)	Modulators PPARδ activates FFA and component of VLDL
		5	Glucocorticoid Receptor (GR) antagonists	Inhibitors of GR preventing the metabolic effects of excessive endogeneous cortisol.
		6	11β-Hydroxysteroid Dehydrogenase Type 1 (11β-HSD1) inhibitors	11β-HSD1 converts 11-keto corticosteroids into 11 β-hydroxy GC's
		7	Ghrelin Analogs and Growth Harmone Seretagogue Receptors (GHS-R) ligands	GHSR-1 has pleictropic effects on many organ system and tissue
D	Insulin sensitivity and inflammation modulators	1	Insulin Receptor Tyrosine Kinase (IRTK) and insulin mimetics	IRTK on autophosphorylation phosphorylate IRS proteins.
		2	Protein Tyrosine Phosphatase 1B (PTP1B) inhibitors	Inhibitors of PTP1B prolong tyrosine phosphorylation status of IRTK, IRS and JAK2
		3	Glycogen Synthase Kinase-3 (GSK3 β) inhibitors	GSK3 β inhibitors inhibits phosphorylation of GS
		4	Inhibitor KappaB Kinase (IKKβ) inhibitors	Inhibitors of IKK- β improved IRS-1/PI3K signal transduction pathway.

Along with above emerging therapeutic targets for diabetes some additional targets are Glucagon receptor antagonists, Resistin, Adipopectine, Triaglyercol lipases, Carnitine palmitoyltransferese-I (CPT1), Cholesteryl ester transfer protein (CETP), ciliary neurotropic factor (CNTF), Insulin receptor, c-Jun N-terminal kinases (JNK), Nitric oxide (NO), Toll-like receptor-4 (TLR4), Interleukin-6 (IL-6), TNF-α, Melanocortin receptor (MC4R), Cannabinoid receptor, Sirtuin-1 (SIRTI) activators, FFAR's other than FFAR1 and FABP's other than aFABP[1].

1.2 Protein tyrosine phosphatase

Approximately 30% of cellular proteins are phosphoproteins and phosphorylation of protein is one of the major post translational modification mechanisms for various cellular regulatory processes and modulation of enzyme activity by generation of recognition motif by removal and addition of phosphoryl moiety for protein-protein interactions[4]. Tyrosine phosphorylation is used for various cellular processes like gene transcription, processing of mRNA, transport of molecules in or out of cells, decision to proliferate versus differentiate, communication between and within cell, the shape and motility of cells. Along with these important processes tyrosine phosphorylation also plays an important role in embryogenesis, organ development, tissue homeostasis and immune system[5].

There are 107 Protein Tyrosine Phosphatases (PTPs) and 90 Protein Tyrosine Kinases (PTKs) in human. Tyrosine phosphorylation plays a dominant role in regulation of many physiological processes in cells. An abnormality in tyrosine phosphorylation leads to pathogenesis of numerous inherited and acquired human diseases from cancer to immune deficiencies. Out of 107 PTP genes, 11 are catalytically inactive, 2 dephosphorylate mRNA and 13 dephosphorylate inositol phospholipids, thus out of 107 PTPs, 81 PTPs have ability to dephophorylate phosphotyrosine. Similarly out of 90 PTKs only 85 are catalytically active; hence the number of active PTPs and PTKs are very similar in number assuming that they have comparable substrate specificities[5,6].

The most significant characteristic of the protein tyrosine phosphatase (PTP) superfamily is conservation of signature motif CX_5R in active site of PTP known as the P-loop or PTP-loop (**Figure-3**)[7].

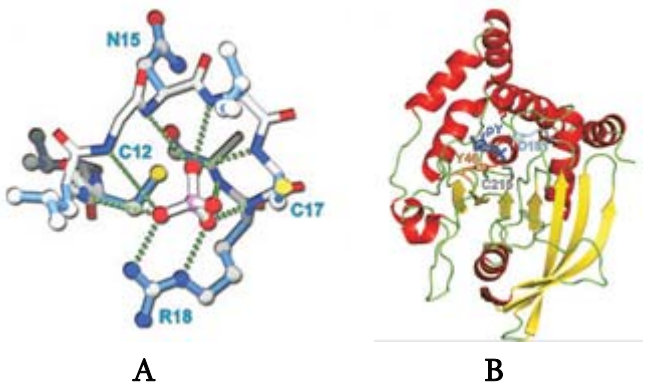

Figure-3: (**A**) Structure of the phosphate-binding loop (P-loop). Stick representation of the consensus signature motif (CX5R) that forms the P-loop present in the active site of PTPs. (**B**) Structure of PTP1B (C215S mutant) in complex with phosphotyrosine (PDB entry 1PTV)[7].

The P-loop of PTP structures are easily superimposed with minor deviation of less than 1Å. The catalytic cysteine, nucleophile in catalysis and arginine involved in phosphate binding remains in close proximity due to structurally conserved arrangement of P-loop which forms a cradle to hold the substrate phosphate group in place for nucleophilic attack. Cysteinyl-phosphate reaction intermediate is formed by nucleophilic attack of cystein thiolate Sγ atom on phosphate ester moiety of the substrate with release of peptidyl tyrosine. The arginine is involved both in stabilization of the reaction intermediate and substrate binding. Substrate binding is followed by conformational changes that culminate with closure of the active site pocket by a conserved and flexible loop of sequence WPD. The amide group in P-loops point towards the interior of the cradle and forms a network of hydrogen bonds to phosphate oxygen. The breakdown of the phosphoenzyme intermediate is facilitated by conserved Ser/Thr residues in the catalytic P-loop which plays an important role in stabilization of thiolate group in transition state. The unique aspartic acid situated in WPD loop participates in catalytic mechanism of PTP reaction and provides general acid and base for catalysis. In the first step during formation of transition state intermediate aspartic acid act as general acid promoting the oxygen of the leaving

group in the tyrosine residue. In the second catalytic step same aspartic acid functions as general base by accepting a proton from the attacking water and promotes hydrolysis of phosphor-enzyme complex and regenerating the free enzyme[8,] (**Figure-4**) [8,4]

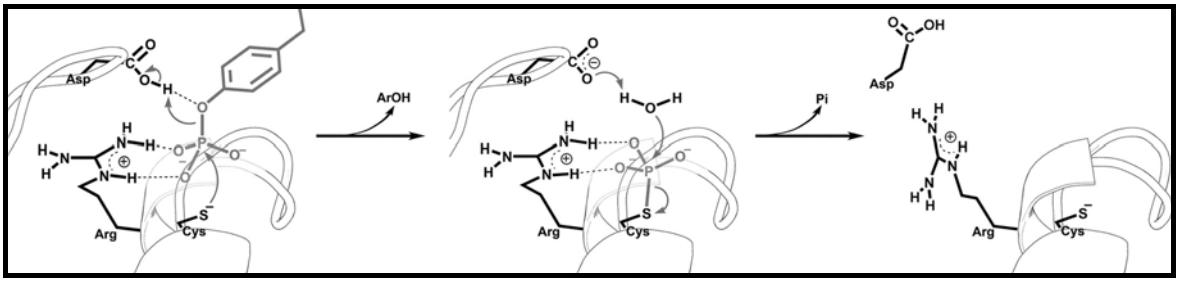

Figure-4: General mechanism of catalysis of PTPs[8,4]

1.2.1 Classification of PTPs

The 107 PTPs can be grouped into four separate families on the basis of amino acid sequence of their catalytic domains, (**Figure-5**). Class I cysteine-based PTPs constitute by far the largest family and contain the 38 well-known "classical" PTPs and 61 "VH1-like (DSPs)" PTPs[9]. The 38 strictly tyrosine specific "Classical PTPs" can be divided into transmebrane, receptor-like enzymes (RPTPs) and the intracellular, non-receptor PTPs (NRPTPs). The 61 VH1-like enzymes are much more diverse and can be divided into several subgroups, 11 MKPs, 19 Atypical DSPs, 3 Singleshots, 3 PRLs, 4 CDC14s, 5 PTENs and 16 Myotubularins which share much less sequence identity with each other than the RPTPs do with the NRPTPs[9]. All Class I PTPs have evolved from a common ancestor. The class II cysteine based PTP in humans is a tyrosine-specific low molecular weight enzyme (1 LMPTP), the origin of which appears to be more ancient than class I PTPs, class II PTPs are structurally related to bacterial arsenate reductases. Class III cysteine based PTPs are tyrosine/threonine-specific phosphatases that most likely evolved from a bacterial rhodanese-like enzyme. They are only represented by the three p80Cdc25 cell cycle regulators. The fourth family of PTPs uses a different catalytic mechanism with a key aspartic acid and dependence on a cation. This family contains the Eya proteins, which were recently discovered to be tyrosine-, or dual serine- and tyrosine-specific protein phosphatases[5,10,11,12].

1.3 Protein tyrosine phosphatase 1B (PTP1B)

Protein Tyrosine Phosphatase 1B (PTP1B) is ubiquitously expressed, well studied non-receptor PTP (NRPTP) anchored to the endoplasmic reticulum (ER) membrane. PTP1B considered a negative regulator of insulin signalling,[6] (**Figure-6**) interacts with the insulin receptor (IR) and removes tyrosine phosphates induced by autophosphorylation in response to insulin binding. PTP1B is also able to dephosphorylate insulin receptor substrates (IRSs) thus, further attenuating and potentially terminating the insulin signaling transduction.

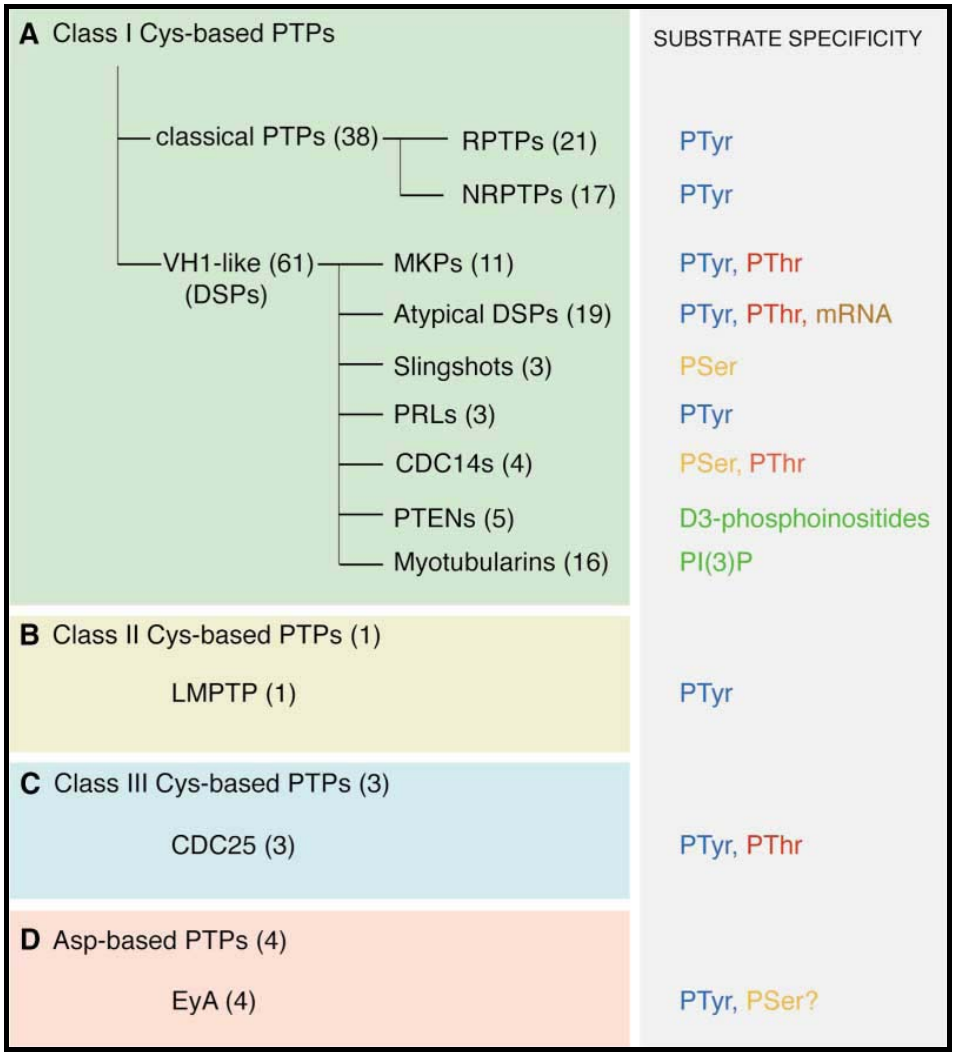

Figure-5: Classification and substrate specificity of PTPs: The PTP families are color coded: class I Cys-based PTPs (green), class II Cys-based PTPs (pale yellow), class III Cys-based PTPs (pale blue), and Asp-based PTPs (pink). The substrate specificity of each group or class of PTPs is listed DSP: Dual-specificity phosphatises, VH-1: Vaccinia virus phosphatase PRL: Prolactin, PTEN: Phosphatase and tensin homologue, LMPTP: Low molecular weight PTP, RPTP: Receptor-like enzymes, NRPTP: Non-receptor PTPs[5].

PTP1B knockout mice ($PTP^{-/-}$) display enhanced sensitivity to insulin, with increased or prolonged tyrosine phosphorylation of IR in muscle and liver. Interestingly, $PTP^{-/-}$ mice are protected against weight gain and have significantly lower triglyceride levels when placed on a high-fat diet. This is unexpected because insulin is also an anabolic factor, and increased insulin sensitivity can result in increased weight gain. PTP1B was subsequently shown to bind and dephosphorylate JAK2, which is downstream of leptin receptor. Thus, the resistance to diet-induced obesity observed in $PTP^{-/-}$ mice is likely to be associated with increased energy expenditure owing to enhanced leptin sensitivity. PTP1B also regulate several other receptor tyrosine kinases (RTKs), including EGFR, PDGFR, CSF-1, etc. In addition to RTKs, PTP1B has other membrane-associated substrates it has been shown to regulate integrin signaling through Src, focal adhesion kinase (FAK) and α-actinin as well as the adaptor protein p130cas. PTP1B can also dephosphorylate the negative regulator p62dok and thus positively influence Ras signaling.

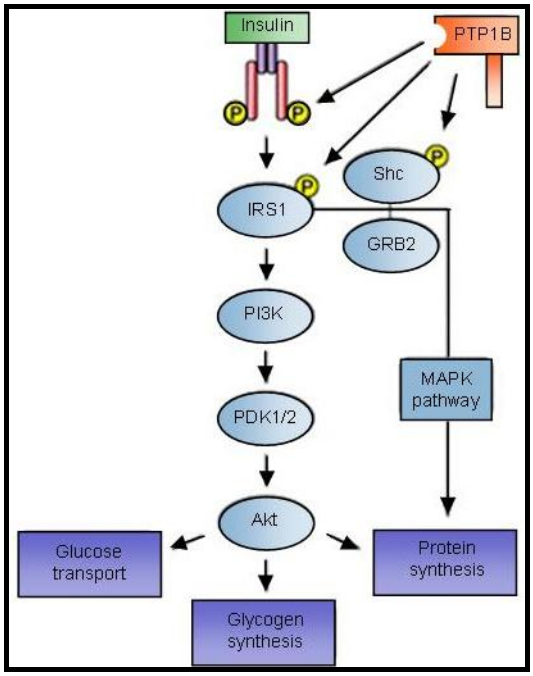

Figure-6: Simplified schematic of the insulin signaling pathway[6].

The oncoprotein p210 Bcr-Abl, which is found in chronic myelogenous leukemia (CML), is another substrate of PTP1B, and certain CML-derived cell lines overexpress PTP1B. Lastly, PTP1B can regulate cytokine signaling through

dephosphorylation of growth hormone, IFN-γ and IFN-α/β, through dephosphorylation of Stat5a/b downstream of prolactin as well as through a direct association with the Epo receptor. The physiological relevance of some of these interactions and substrates still remains to be explored.

1.3.1 Structural features of PTP1B

The secondary structure of PTP1B includes a central, highly twisted β sheet composed of eight β strands forming mixed β sheet with four parallel strands flanked by antiparallel ones. Six α helices surround the central sheet, four on one side and two on the other. The active site is located within a crevice, 9 Å deep for the tyrosine-specific PTPs and 6 Å deep for the dual–specificity phosphatases, thus providing selectivity for phosphotyrosine-containing protein substrates. The signature motif comprises of 11 residues VHCSXGXGR(T/S)G that form the PTP-loop between the C-terminus of the central β10 strand and the α4 helix is located at the bottom of catalytic site. Cys215 and Arg221 residues in the PTP-loop are most vital for catalysis. Another conserved loop is phosphotyrosine-recognition loop with the conserved motif KNRY (residue 43-46). This loop determines the depth of the active site cleft and interacts through its tyrosine residue with the aromatic ring of the phosphotyrosine in the substrate. A third conserved loop is the WPD loop (residue 179-181). PTP1B contains additional C-terminal helix α7, not present in other PTP structures. This is particularly an important regulatory element for the catalytic activity of PTP1B as it stabilizes closure of WPD-loop through its interaction with helices α3 and α6. On and near the WPD loop are key residues that function in PTP1B catalysis. Asp 181 and Gln 262 become especially important in the second part of the reaction. These structural features of PTP1B provide for the chemistry of dephosphorylation, detailed below:

A. Dephosphorylation of tyrosine-phosphate residue

The reaction starts when a phosphorylated tyrosine residue enters the deep, active site cleft of PTP1B molecule (**Figure-7**), the base of which is the PTP loop. Tyr46 and Val49 of the recognition loop facilitate this entry. Phosphotyrosine is an amphipathic molecule. The phosphorylated end of the tyrosine is polar, but the phenyl ring is non-

polar and would normally be repelled from a polar catalytic site. Tyr46 and Val49 provide a non-polar pocket for the phenyl ring of the phosphotyrosine substrate while the phosphorylated end is securely placed in the catalytic cleft. When the substrate enters the catalytic site, a major conformational change occurs in the WPD loop. The loop closes over the phenyl ring of the tyrosine residue, holding it in place and further positioning it so that a subsequent nucleophilic attack may occur. At the same time, Asp181 is moved in close to the tyrosine phosphate so it can act as an acid during the reaction. Binding also occurs within the PTP loop; Arg221 shifts to optimize its connection with the phosphate attached to the tyrosine residue. The slight shift of Arg221 increases binding with Pro180, Trp179, and Phe182. All these interactions lead to a stable closed conformation for the WPD loop. The phosphorylated tyrosine residue is situated in such a way that the phosphorus atom and the gamma sulfur atom of Cys215 are juxtaposed. This is essential for catalysis because the Cys215 residue of the PTP loop will remove the tyrosine phosphate and store it briefly as an intermediate. First, Asp181 adds a proton (hydrogen) to the oxygen of tyrosine. This neutralizes the tyrosine and it is then free to diffuse away from the catalytic cleft. The captured phosphate then binds to the sulfur of Cys215, thereby forming the cysteinyl-phosphate intermediate.

B. Dephosphorylation of cysteinyl-phosphate intermediate

The WPD loop retains its closed conformation after the Tyrosine residue diffuses away from the enzyme because amino acids Arg221, Pro180, Trp179, and Phe182 maintain interactions with the phosphate group bound to Cys215. The phosphate is removed from the cysteine via a nucleophilic attack of a water molecule. Although this attack is kinetically unfavorable because of steric repulsion, Gln262 and Asp181 (the amino acid that protonated the oxygen of the tyrosine residue) neutralize and position the H_2O (W2) for attacking the cysteinyl-phosphate intermediate. Mutation of either Gln262 or Asp181 blocks this step of the reaction. W2 breaks the bond between the phosphate and the cysteine. The phosphate then binds to W2 forming a water

phosphate complex. The enzyme then returns to a standard conformation, ready to accept another phosphorylated tyrosine into the active site.

The structure of PTP1B also reveals the presence of secondary phosphotyrosine-binding sites within their catalytic domains. These secondary substrate-binding sites are represented by a positively charged pocket located close to the active site. In PTP1B, the secondary site is formed by Arg24, Arg254, Met258 and Gly259 and plays the important role of providing specificity for PTP1B action. This is illustrated by the fact that a physiological substrate of PTP1B, the insulin receptor kinase, contains a tandem of phosphotyrosine residues (1162 and 1163) and interacts with PTP1B in a characteristic bidentate mode, pTyr1162 is recognized and selectively dephosphorylated by the active site, whereas pTyr1163 is bound to the secondary binding site of PTP1B. A different type of allosteric regulation of the catalytic activity was also reported, benzbromarone derivative are non-competitive inhibitor of PTP1B.

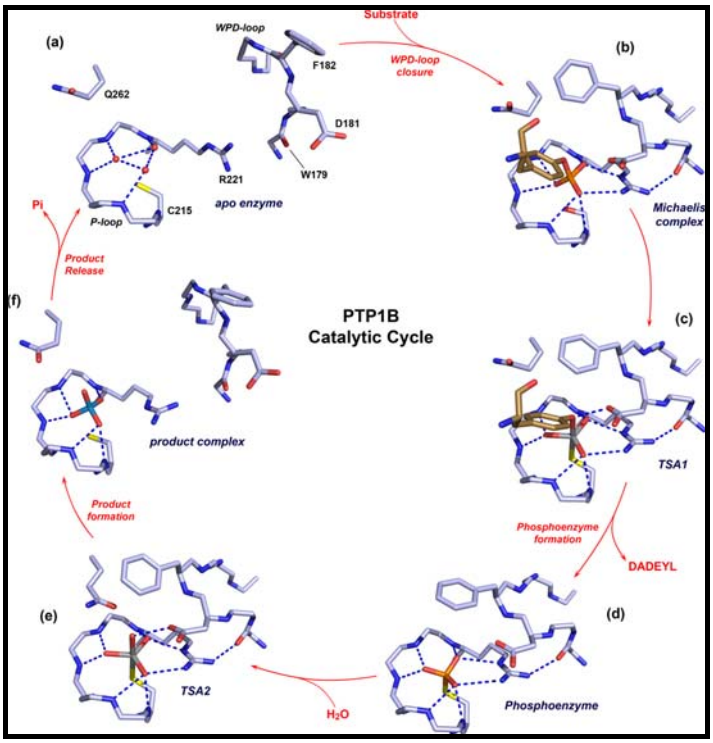

Figure-7: Crystal structures along the pathway of the reaction catalyzed by PTP1B.

This binding site is located ~2 Å from the active site and is form by helices α3 and α6. The inhibitory effect seems to result from blocking the interaction between helices α7 and α3-α6, present in closed form of PTP1B, thus preventing closure of the WPD-

loop. PTP1B lacking the α7 is four folds less active than the native form. This finding provides additional support for the significance of helix α7 in controlling the catalytic activity of PTP1B